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Micromanipulation of Chromosomes Reveals that Cohesion Release during Cell Division Is Gradual and Does Not Require Tension

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Summary

In mitosis, cohesion appears to be present along the entire length of the chromosome, between centromeres and along chromosome arms. By metaphase, sister chromatids appear as two adjacent but visibly distinct rods [1–4]. Sister chromatids separate from one another in anaphase by releasing all chromosome cohesion. This is different from meiosis I, in which pairs of sister chromatids separate from one another, moving to each spindle pole by releasing cohesion only between sister chromatid arms [5–8]. Then, in anaphase II, sister chromatids separate by releasing centromere cohesion. Our objective was to find where cohesion is present or absent on chromosomes in mitosis and meiosis and when and how it is released. We determined cohesion directly by pulling on chromosomes with two micromanipulation needles. Thus, we could distinguish for the first time between apparent doubleness as seen in the microscope and physical separability. We found that apparent doubleness can be deceiving: Visibly distinct sister chromatids often cannot be separated. We also demonstrated that cohesion is released gradually in anaphase, with chromosomes looking as if they were unzipped or pulled apart. This implied that tension from spindle forces was required, but we showed directly that no tension was necessary to pull chromatids apart.

Results and Discussion

Chromosome Cohesion Is Released Gradually over the Length of a Chromosome in Anaphase and Not before

We wanted to determine where cohesion was present on the chromosome, where it was absent, and whether apparent doubleness of chromatids correlated with absence of cohesion during cell division. Apparent doubleness and some separation between chromatids has been observed prior to anaphase [1] and in cells treated with colchicine to inhibit formation of the mitotic spindle [1, 2]. However, fine connections between the chromatids could not be ruled out. Two microneedles were used to pull chromatids apart to determine directly whether there was some cohesive force holding chromatids together. If, on release from the needles, the chromosomes returned to their position adjacent to one another, cohesion was present. If they stayed apart after the pulling, cohesion was absent. All experiments described were done in cells of the grasshopper *Melanoplus sanguinipes* (Fabricius). Cells were cultured as previously described [9].

Mitosis

Ten spermatogonia were observed from mitotic metaphase through late anaphase. Two microneedles were used to pull sister chromatids apart (Figure 1; 0 and 1 min). Sister chromatids in metaphase cells were visibly distinct from one another, with some apparent space between sister chromatids (Figure 1; 0 and 2 min). Nevertheless, they returned to their position adjacent to one another when the pulling by the microneedles was released (Figure 1; 2 min). This shows that they were not actually physically separable. After anaphase onset (Figure 1; 7 min), chromatids began to separate at their centromeres, but they were not fully separable along their entire length: They snapped back together at their ends after separation by the microneedles (Figure 1; 7 and 8 min). Eventually, by late anaphase, chromatids were fully separated (Figure 1; 11 and 16 min).

Meiosis I

Two microneedles were used to pull chromatids apart in a metaphase I spermatocyte (Figure 2; 0 and 1 min, arrow and arrowhead). When pulling by the two needles was released, homologs returned to their previous position (Figure 2; 1′ min, arrow and arrowhead), indicating that there was cohesion between chromosome arms. At anaphase onset, homologous chromosomes began to separate from one another in the region between homologous centromeres (Figure 2; 17 min). They continued to separate toward the ends of the chromosomes, but some cohesion between arms was still present (Figure 2; 17–20 min). Finally, arms were completely separate from one another (though sister centromeres remained together) (Figure 2; 22 min). Ten similar cells were examined, and all displayed this behavior.

Meiosis II

A metaphase II chromosome (Figure 3; 0 min, arrows) was manipulated with two needles to separate sister chromatid arms (Figure 3; 1 min, arrows). Pulling was released, and the chromatid arms remained separated (Figure 3; 15 min, arrows), although it was not possible to separate sister centromeres (Figure 3; 31 min, arrows). The separation (Figure 3; 30 and 41 min, arrows) and release (Figure 3; 31 and 42 min, arrows) were repeated twice, with the same result. The cell entered anaphase (Figure 3; 44 min), and sister chromatids fully separated (Figure 3; 47 and 62 min, arrows).

These microneedle experiments show that in mitosis and meiosis I, chromosome cohesion is present along the entire length of the chromosome until anaphase onset, even though chromosomes are visibly double by metaphase (Figure 1, 2 min; Figure 2, 1′ min). This reveals an important distinction between visible doubleness and physical separability and has implications for the molecular basis of cohesion. There are two contributors to cohesion: a protein complex called cohesin, which links chromatids together, and tangles or catenations between sister DNA molecules. The cohesin complex con-

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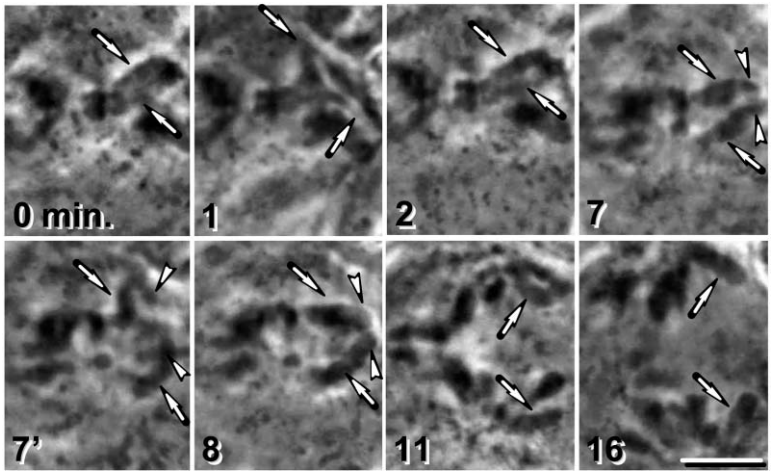

Figure 1. Location of Cohesion and Timing of Its Release in Mitotic Chromosomes

Two micromanipulation needles pulled sister chromatids of a mitotic (metaphase) chromosome apart (0 and 1 min, arrows). Sister chromatids returned to their position adjacent to one another after release (2 min, arrows). In early anaphase (7 min), chromatids (arrows) were again pulled apart with two needles, and the ends of the chromatids distal to the centromere (arrowheads) again returned to their side-by-side position (7′ and 8 min). At late anaphase, sister chromatids were completely separated (11 and 16 min, arrows). The scale bar represents 5 μm.

sists of four subunits, with some common to all cell division types and some that are mitosis-specific or meiosis-specific [7]. For mitotic chromosomes, sister chromatids are linked extensively along their length during DNA replication. Upon chromosome condensation in prophase, chromosomes appear to release all of their cohesin along the chromosome arms, but the complex is retained between sister centromeres [11, 12]. This has been shown both in *Drosophila* and in mammalian cell culture, and we have verified this in grasshopper mitotic cells by immunofluorescence staining of Smc3, a component of the cohesin complex (data not shown). However, in *Xenopus* extracts and in cultured mammalian cells, a small amount of epitope-tagged cohesin can be

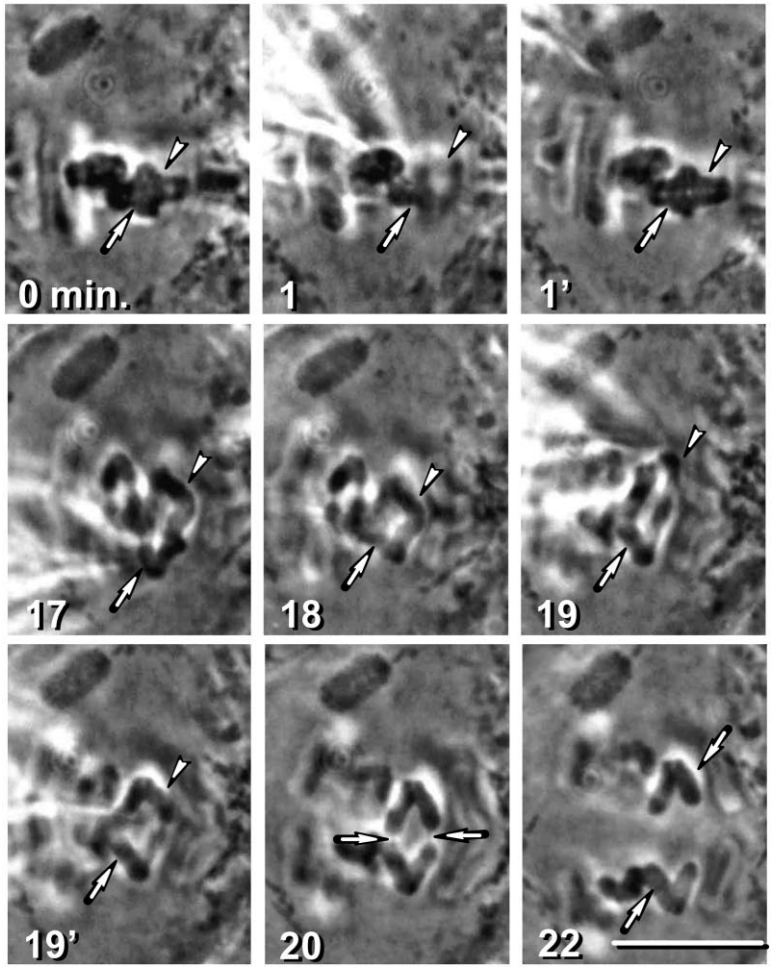

Figure 2. Location of Cohesion and Timing of Its Release in Meiosis I Chromosomes

A metaphase I chromosome (0 min, arrow) was manipulated with one needle pulling on one homolog (1 min, arrow) and the other pulling the other homolog in the opposite direction (1 min, arrowhead). Upon release from the pulling by the two needles, homologs returned to their previous position (1′ min, arrow and arrowhead). Homologs were twice more pulled apart as the cell underwent anaphase (pulling: 17 and 19 min, arrow and arrowhead; release: 18 and 19′ min, arrow and arrowhead). Strands between homologs (20 min, arrows) were absent by late anaphase, when cohesion only between sister centromeres was present (22 min). The scale bar represents 10 μm.

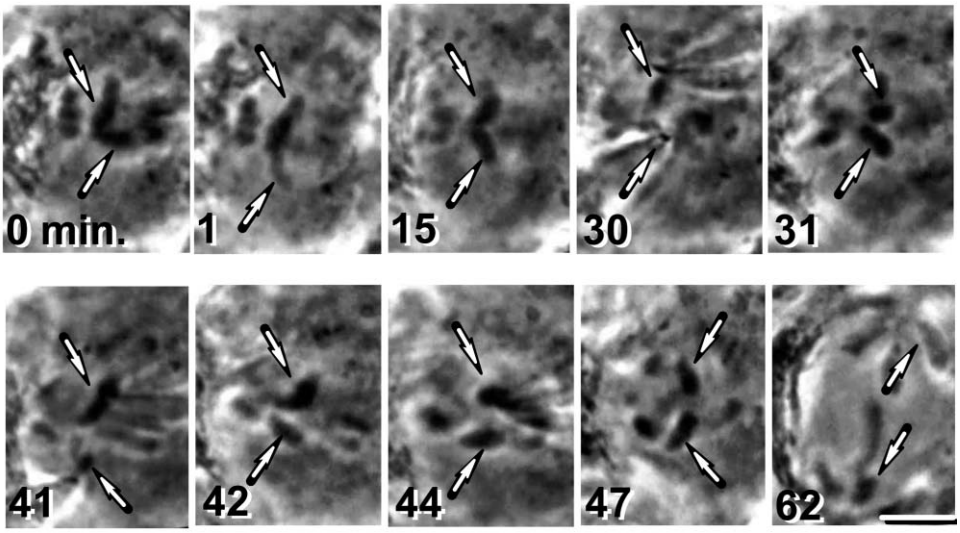

Figure 3. Location of Cohesion and Timing of Its Release in Meiosis II Chromosomes

Two microneedles were used to pull chromatids apart in a metaphase II spermatocyte (0 and 1 min). Sister chromatid arms stayed well separated after multiple separations (1, 30, and 41 min, arrows) and releases (15, 31, and 42 min, arrows), although they were held together at centromeres. Chromatids were fully separated from one another at anaphase (44 min, arrows) and segregated to opposite poles (47 and 62 min, arrows). Ten cells were examined, and all displayed this behavior. Part of the lower chromatid distal to the centromere in the 42 and 44 min images is out of focus. The scale bar represents 5 μm.

visualized along chromosome arms in metaphase [10, 13]. At anaphase, a component of the cohesin complex, Mcd1/Rad21/Scc1, is cleaved by the protease separase, which leads to the complete loss of the complex from chromosomes and loss of cohesion [14].

Our results in mitotic chromosomes reveal that something must be holding chromatid arms together through late anaphase, even though the levels of cohesin are much reduced or even absent by mitotic metaphase. Either there is some small amount of functioning cohesin along chromosome arms, and it is sufficient for holding them together but not sufficient to be seen, or DNA catenations linking chromatid arms hold the sister chromatids together.

Cohesion is present between sister chromatids in meiosis through anaphase I, which corresponds with the presence of cohesin complexes on chromosomes in meiosis I. Cohesion is released gradually along chromosome arms in both mitosis and meiosis I. In anaphase I, Rec8 (a homolog of Mcd1/Rad21/Scc1 and a member of the meiotic cohesin complex) is cleaved, the complex is released from chromosome arms, and cohesion is lost from the arms [6, 15]. The centromeric pool of cohesin is protected from Rec8 cleavage by a protein called MeiS332 in *Drosophila* or its homolog Sgo1 in yeast [16, 17]. Release of cohesin along chromosome arms leads to, and is required for, loss of arm cohesion and homolog separation [16]. In meiosis II, cohesin is present only at the centromere [6, 15] until anaphase, when all cohesion is lost.

In meiosis I, we find that release of cohesion starts near the centromere and proceeds toward the chromosome ends distal to the centromere (Figures 1 and 2) but is retained at the centromere itself. These results verify and extend those of Suja et al. [18], who showed that telomeres separate last in meiotic anaphase. However, they could not show that telomeres were actually physically connected to one another, which we have demonstrated. It is not entirely surprising that release of cohesion is not simultaneous along the length of the chromosome because separation of telomeres in anaphase has been shown to be regulated differently from the rest of the chromosome [6, 19–22]. Here, though, we add that separation is gradual over the whole length of the chromosome—it is not just that the telomeres are separated last. Similar gradual separation has been previously observed in mitosis in cultured mammalian cells [23]. Mitotic and meiosis I grasshopper chromosomes always appear to separate with chromatids looking as if they "unzip" from one another, as if they were being pulled apart by spindle-associated tension. We were curious whether tension was required to separate chromatids in anaphase.

Chromosomes Do Not Need to Be Attached to a Spindle to Lose Cohesion

One way to assess whether tension is required to fully separate sister chromatids in anaphase is to eliminate the kinetochore. This has been done previously in grasshoppers with X-rays to generate chromatid fragments lacking kinetochores. The fragments were often visibly double, but fine connections between sister fragments were apparent [24, 25]. This stickiness, however, may have been caused by exposure to X-rays. To address the question of whether separation could be achieved without tension, we used a laser microbeam (365 nm; Micro Point Laser System, Photonic Instruments, Arlington Heights, IL) to cut a chromosome in a mitotic metaphase neuroblast cell so the kinetochore was separated from the fragment under observation (Figure 4; 0 and 1 min, arrow). Before anaphase, sister chromatid fragments were connected (Figure 4; 4 min). After anaphase

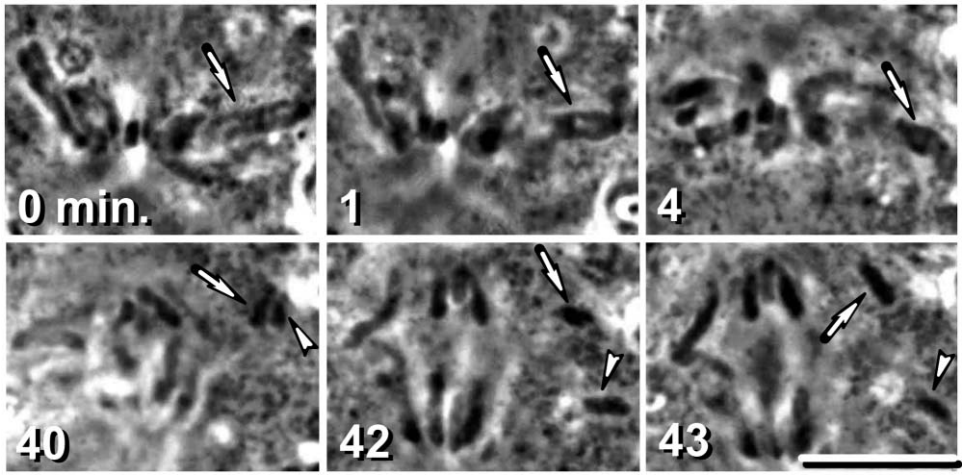

Figure 4. Cohesion Is Released in Anaphase between Chromatid Fragments Lacking Kinetochores

A mitotic (prometaphase) chromosome in an embryonic neuroblast (0 min, arrow) was cut with a laser microbeam so that the remaining fragment had no kinetochores and could not attach to the spindle (1 and 4 min, arrows). In anaphase, sister chromatid fragments began to separate (40 min, arrow and arrowhead). By late anaphase, pushing the upper chromatid fragment with a single microneedle (42 and 43 min, arrow) did not alter the position of its sister chromatid fragment (42 and 43 min, arrowhead). The scale bar represents 10 μm.

onset, sister chromatid fragments became visibly distinct (Figure 4; 40 min, arrow and arrowhead) and were physically separable with a single microneedle soon after (Figure 4; 42 and 43 min). This indicates that the chromatid fragments were fully separable, even though they were unable to attach to the spindle. These results verify by micromanipulation the results of Liang et al. [26], who showed separation of laser-generated sister chromatid fragments in anaphase.

An entirely different test of whether tension is required for chromatid separation avoids use of a microbeam and is applicable to meiosis as well as mitosis. In these experiments, we take advantage of the fact that there is a period of approximately ten minutes in metaphase, just prior to anaphase onset, during which chromosomes of any division type can be detached from the spindle without delaying anaphase. Such detachment is genuine—all previous kinetochore-microtubule attachments are lost [27]. Thus, the cell enters anaphase with a chromosome that is not feeling any of the forces that accompany attachment to the spindle. We examined these chromosomes to determine whether they lost chromosome cohesion during anaphase in meiosis I, meiosis II, and mitotic cells.

In a meiosis I spermatocyte, one bivalent was detached from a late metaphase I spindle (Figure 5; 0 and 1 min, arrows). The chromosome was kept from reattaching to the spindle by using a single microneedle to push it around (Figure 5; 7, 8, and 11 min, arrows). Chromatids became visibly distinct from one another after anaphase onset (Figure 5; 11 min, arrows). Finally, the homologous chromosomes were fully separable, and pushing one pair of chromatids with a single microneedle did not affect the position of its partner (Figure 5; 18 min, arrows). Similar results were obtained for eight manipulated bivalents in eight different cells. The same experiment was done in mitosis and meiosis II with identical results: Chromatids release cohesion in the absence of tension (data not shown). This is interesting in light of previous results in the study of topoisomerase II.

Catenations between sister DNA duplexes contribute to chromosome cohesion and occur as an inevitable result of DNA replication. Topoisomerase II is required to disentangle sister chromatids by catalyzing a double-strand break in one DNA duplex, allowing another to pass through the break, and then resealing the break [5, 28]. The importance of catenations as a cohesive force is not established. Certainly they must be resolved by anaphase, but we do not know how much they contribute to the physical cohesion between chromatids. The activity of topoisomerase II is required for proper chromosome separation in anaphase [28–30]. There is no directionality to this process, however, and topoisomerase II can just as easily entangle as disentangle [28]. For decatenation to be favored, the sister DNA molecules must be separated from one another after topoisomerase II activity. Most of this resolution occurs at chromosome condensation in prophase, in association with the machinery of chromosome condensation [31, 32]. However, topoisomerase II activity is definitely required at anaphase onset for chromosome separation because application of topoisomerase II inhibitors in metaphase prevents resolution of the remaining catenations in anaphase, and chromatids fail to separate fully [28–30]. Our results show that the pulling provided by anaphase movements on the spindle does not drive decatenation in anaphase because chromosomes off the spindle in any division have chromatids or homologs that are fully separable without any further pulling on our part (one chromatid or homolog can simply move independently of the other [Figures 4 and 5]). If the mitotic forces and anaphase movement do not give directionality to topoisomerase II, what does? One possibility for the separator is the loss of cohesin molecules from the chromosome. Sister chromatids may be held together so tightly by cohesin that its loss leads to chroma-

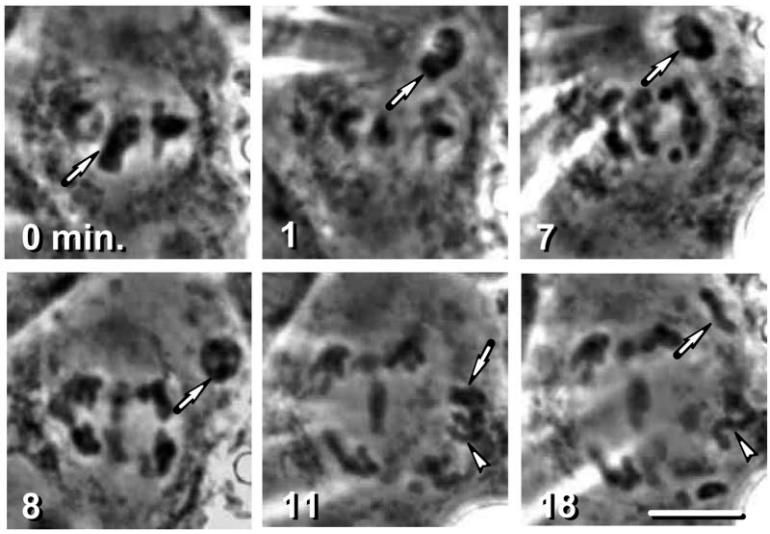

Figure 5. Cohesion Is Released along the Arms of a Meiosis I Chromosome in Anaphase, Even if the Chromosome Is Not Attached to the Spindle

All manipulations were done with a single microneedle. A metaphase I chromosome (0 min, arrow) was detached from the spindle (1 min, arrow) and kept from reattaching even as the cell progressed through anaphase (7 and 8 min, arrow). By mid-anaphase, homologous chromosomes began to appear separate (11 min, arrows). Later, when one half-bivalent was pushed with a single microneedle (18 min, arrow), its homolog remained in its previous position (18 min, arrowhead). The scale bar represents 10 μm.

tids or homologs falling apart in a manner that also drives decatenation. Of course, this assumes that cohesin is actually present along chromosome arms in mitosis, in which no apparent cohesin staining occurs in grasshopper cells. Alternatively, there could be some protein driving decatenation at anaphase onset. Because topoisomerase II is known to interact with certain proteins required for condensation [31, 32], it is possible that these proteins are modified at anaphase to give the final push to decatenation of sister chromatids.

We have resolved several sticky points in the study of chromosome cohesion. We showed that visible doubleness does not necessarily mean physical separability and that chromatid separation can happen in the absence of forces associated with spindle attachment. The question for the future is, what is it that links chromatids through late metaphase, and how is the linkage released gradually rather than simultaneously along the length of the chromosome? Finally, how are the last catenations between chromatids resolved in an anaphase chromosome when anaphase chromosome movement is not necessary for chromosome separation?

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